Pesticide Biochemistry and Physiology 99 (2011) 111–117

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Elevated antioxidant response and induction of tau-class glutathione S-transferase after glyphosate treatment in *Vigna radiata* (L.) Wilczek

Mahesh Basantani [a], Alka Srivastava [a,*], Somdutta Sen [b]

[a] *In Vitro Culture and Plant Genetics Unit, Department of Botany, University of Lucknow, Lucknow, India*
[b] *The Centre for Genomics Application, New Delhi, India*

ARTICLE INFO

Article history:
Received 9 July 2010
Accepted 9 November 2010
Available online 24 November 2010

Keywords:
Affinity chromatography
Herbicide
Glyphosate
Vigna radiata
Tau GST
Antioxidant response

ABSTRACT

Glyphosate (N-(phosphonomethyl) glycine) is a broad-spectrum herbicide, acting on the shikimic acid pathway inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), thus obstructing the synthesis of tryptophan, phenylalanine, tyrosine and other secondary products. It has also been reported to generate oxidative stress which influences the antioxidant response of target plants. The effect of glyphosate application on total protein, CAT, POD and GST activities was investigated and elevated expression of the oxidative stress enzymes was obtained after glyphosate treatment.

Tau-class GSTs are plant-specific, and are chiefly involved in xenobiotics and oxidative stress metabolisms. Many herbicides and safeners have been known to selectively induce tau-class GSTs in different plant species. Here we also report the induction of tau-class GSTs after glyphosate treatment in the seedling roots of two *Vigna radiata* varieties (PDM11 and PDM54). GSH-agarose affinity chromatography and mass spectrometry revealed that the tau-class GSTs induced in the two varieties were different; the tau-class GSTs present in the untreated controls were also different in the two varieties. The present study highlights the elevated antioxidant response, the induction of tau-class GST and the genotypic variation in the type of tau-GST in control and glyphosate treated varieties of *V. radiata*.

1. Introduction

Glyphosate is used to control several weeds, which include grasses, sedges and other broad-leaved weeds. It acts as a potent inhibitor of shikimic acid pathway for the biosynthesis of aromatic amino acids. It is a competitive inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) with respect to phosphoenolpyruvate (PEP) and noncompetitive with respect to shikimate-3-phosphate (S3P) [1]. The inhibition of EPSP synthase leads to killing of plants due to scarcity of the three aromatic amino acids.

Glyphosate is considered to be less toxic to animals due to the absence of shikimic acid pathway in animals, but certain studies have shown the detrimental effects of this herbicide on animal systems: it leads to cell cycle dysfunction [2], inhibits global transcription [3] and has teratogenic potential [4].

Catalases (CAT, EC 1.11.1.6) involved in herbicide tolerance, or an increase in CAT activity during herbicide exposure, have been reported from several plant species [5–7]. A few earlier reports too have shown an increase in CAT activity in *Vigna radiata* plants after herbicide exposure. 2-Benzoxazolinone (BOA) was found to cause oxidative stress in mung bean plants, which responded by an increase in the activity of ROS scavenging enzymes like CAT and superoxide dismutase (SOD, EC 1.15.1.1) in the root and leaf tissues [8]. Sergiev et al. [9] demonstrated that catalase activity was increased after 6 and 10 days of glyphosate application in maize plants. Cañal et al. [10] could demonstrate the activation of different peroxidase (POD, EC 1.11.1.7) isozymes at different glyphosate concentrations in *Cyperus esculentus* L. plants.

Glutathione S-transferase (GST, E.C. 2.5.1.18) act as major phase II detoxification enzymes like glycosyl transferases. The GST gene family in plants is represented by eight distinct classes: seven of these (phi, tau, zeta, theta, lambda, DHAR, TCHQD) are soluble, and one is microsomal [11]. The phi and tau classes are plant specific and the most abundant. Several members from both the classes involved in diverse metabolic processes are very well characterized from different plant species [12]. GSTs from both the phi and tau classes are induced upon exposure to herbicides and protect plants from herbicide injury. Tau-class GSTs have been mainly studied in relation to their role in xenobiotics and oxidative stress metabolisms. Roxas et al. [13] emphasized upon the role of tau-class GSTs in chilling and oxidative stress. They showed that tobacco seedlings overexpressing a tau-class GST, having a high GPOX activity, were more tolerant to oxidative stress. This was one of the first reports demonstrating the importance of tau-class GSTs in oxidative stress metabolism. Kilili et al. [14] identified five tau-class GSTs playing major role in oxidative stress in tomato. Moons [15] showed that tau-class GSTs are induced upon exposure to heavy metals as well. Tau GSTs

* Corresponding author.
E-mail address: alkasrivastava@hotmial.com (A. Srivastava).

0048-3575/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2010.11.007

involved in herbicide metabolism have been identified in *Triticum tauschii* [16], soybean [17], wheat [18] etc.

The effect of glyphosate on plants, its toxicity and tolerance, has been studied mainly in relation to EPSPS. There is dearth of literature on the role of GSTs in glyphosate metabolism. However, GST activity is found to be induced by glyphosate, and there are only a few reports demonstrating this induction [19,20,9]. This increase in GST activity enhances the tolerance of crop plants towards glyphosate. Moreover, the studies carried out so far have not shed any light on the class of GST involved in glyphosate metabolism. The aim of the present study was to identify the effects of glyphosate herbicide on *V. radiata*, to understand the role of antioxidant enzymes in the detoxification of glyphosate, and, most importantly, to purify and recognize the class of GSTs induced after glyphosate treatment. The activity of antioxidant enzymes CAT and POD was also measured. Moreover, the glyphosate-induced GST has been purified in order to be assigned to a particular class by using mass spectrometry analysis of the purified protein.

2. Materials and methods

2.1. Herbicide treatment

The seeds of the *V. radiata* varieties PDM11 and PDM54 were procured from Indian Institute for Pulse Research, Kanpur. The chemicals used were purchased from HiMedia, Mumbai, India. GSH-agarose and protein molecular weight marker (Medium Range) were supplied by Bangalore Genei, Bangalore, India. The seeds were soaked for 24 h in increasing isopropylamine glyphosate (Monsanto, India) concentrations prepared in water.

The seeds of both the varieties were treated with glyphosate solution (20 mL) at concentrations 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM. Control seeds were treated with water only. After soaking, the seeds were placed on moist filter paper in petridishes for germination. The germination and survival percentages were observed and seedling root length was measured. The survival percentage was measured after 12 days of germination. The roots were harvested after 12 days for protein extraction, GST purification and enzyme assays.

2.2. Protein isolation and estimation

After germination, the seedling roots were harvested for protein extraction and GST purification. The tissue was ground to a fine powder in liquid nitrogen and homogenized in the extraction buffer (0.2 M Tris–HCl pH 7.8, 1 mM EDTA, 20% glycerol and 2 mM PMSF). The protein samples were quantified by the Bradford method [21], using BSA as the standard.

2.3. GST enzyme assay

The GST activity was measured spectrophotometrically according to Habig et al. [22]. The final assay mixture consisted of 50 mM phosphate buffer pH 6.5, 1 mM CDNB, 1 mM GSH, 0.5 mM EDTA and the seedling root extract containing 100 μg protein. The final reaction volume was made to 2.5 mL with water. The reaction was started by the addition of the root extract. The reaction was monitored spectrophotometrically at 340 nm. The GST activity was expressed as as μmol min^{-1} mg^{-1} protein. It was the measure of DNP–GS complex formed.

2.4. CAT enzyme assay

CAT activity was measured according to Euler and Josephson [23]. A 2.5% protein extract (in the extraction buffer) was prepared. 2 mL citrate phosphate buffer (pH 7.0), 1 mL water and 1 mL enzyme extract were taken in two sets of test tubes. One of the sets was labeled blank and the other as sample. In the sample, 1 mL H_2O_2 was added and exactly after 10 min the reaction was stopped by adding 2 mL 4 N H_2SO_4. In the blank, 2 mL 4 N H_2SO_4 was added first and then 1 mL H_2O_2 was added. The reaction mixtures were titrated against 0.01 N KMnO4 till the end point was (light pink) reached. The catalase activity was expressed as mL H_2O_2 decomposed g^{-1} fresh weight of tissue.

2.5. POD enzyme assay

POD activity was estimated by a modified method of Luck [24]. 2.5% protein extract was prepared. Two milliliters citrate phosphate buffer (pH 6.0), 1 mL H_2O_2 and 1 mL p-phenylenediamine were taken in two sets of test tubes; one was blank and the other was labeled sample. In the sample, 1 mL enzyme extract was added and allowed to stand for 10 min. After 10 min 2 mL 4 N H_2SO_4 was added to stop the reaction. In the blank, 2 mL 4 N H_2SO_4 was added and then 1 mL enzyme extract was added. All the reaction mixtures were allowed to stand for 1 h at room temperature in the dark. After 1 h A485 was measured with the spectrophotometer. The enzyme activity was expressed as the difference in OD between blank and sample per gram fresh weight (ΔOD g^{-1} fresh wt).

2.6. GSH-agarose affinity chromatography

GST enzyme was purified according to DeRidder et al. [25]. The tissue was ground to a fine powder in liquid nitrogen and homogenized in the extraction buffer containing 20 mM Tris–HCl pH 7.8, 1 mM EDTA and 5 mM DTT (buffer A). The homogenate was filtered through two layers of cheesecloth and centrifuged at 14,000 rpm for 20–30 min at 4 °C. GST purification using GSH-agarose affinity chromatography was conducted according to DeRidder et al. [25] with slight modifications. The GSH-agarose affinity column was run using gravity flow. The affinity column was first equilibrated with five column volumes of buffer A. After equilibration the protein extract was loaded on the matrix. After protein loading, the matrix was washed with 3–5 column volumes of buffer A. GST was eluted with three column volumes of buffer B (20 mM Tris–HCl pH 7.8, 1 mM EDTA and 10 mM GSH). The purified GST protein was run on the 12% polyacrylamide gel (Fig. 1). The band corresponding to GST (molecular weight approx. 27 kDa) was cut from the gel and submitted to The Centre for Genomic Applications (TCGA), Proteomics Facility, New Delhi, for identification of the protein by mass spectrometry.

2.7. Mass spectrometry

The protein band corresponding to 27 kDa was cut from the gel and subjected to in-gel trypsin digestion and then MS/MS analysis. The digestion and MS/MS analysis were carried out at the proteomics facility of The Centre for Genomic Application (TCGA), New Delhi.

The sample preparation was accomplished according to Shevchenko et al. [26] with slight modifications. The gel piece was first washed with 500 μL water. The silver stained gel piece was destained by immersing them in 1:1 solution of potassium ferricyanide (15 mM) and sodium thiosulphate (50 mM) for 10 min. After the destaining the gel piece was washed twice with 500 μL water to remove reducing agents. The gel piece was equilibrated with 200 mM/L of NH_4CO_3, and reduced using 150 μl of 10 mM DTT in 100 mM ammonium bicarbonate, and 5% acetonitrile (ACN) for 1 h at 55 °C. The gel piece was rehydrated with 100 mM NH_4CO_3 for 10 min. and then dehydrated in ACN for 20 min.